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In our previously report, we demonstrated that the systemic administration of murine endothelial cells expressing human interleukin (IL)-2 gene (hIL-2-GMEC) mediated significant reductions of established pulmonary macrometastases from breast cancer. In the present study, we analyzed hIL-2-GMEC-targeted and hIL-2-GMEC-negative breast tumor metastases to determine the immune effector cells that may be involved in hIL-2-GMEC-mediated regression of breast cancer metastases.

hIL-2-GMEC-negative metastases were infiltrated by granulocytes and macrophages. These cells were confined to the edges of the tumors. In contrast, rhIL-2-positive tumors had an extensive cellular infiltrate made up of granulocytes (mainly neutrophils), macrophages, CD4+ and CD8+ lymphocytes. More importantly, these cells were present around and within individual metastasis. These results suggest that both non-specific and specific immune effector cells may be involved in hIL-2-GMEC-mediated regression of breast cancer metastasis.

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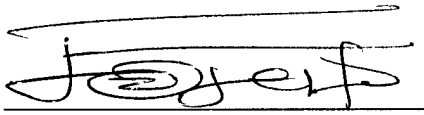

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1. INTRODUCTION

Recurrence and metastatic dissemination of breast cancers account for a significant morbidity and mortality in women, and effective means of treating this subset of patients remain elusive. Our long term goal is to develop an effective and safe systemic gene therapy for metastatic breast cancer. In our earlier reports, we demonstrated that endothelial cells stably express herpes simplex *lacZ* (*LacZ*) and human interleukin (hIL)-2 transgenes *in vitro* and *in vivo* (1). We have shown further that systemically-administered, *lacZ* or IL-2 gene modified endothelial cells (*lacZ*-GMEC or hIL-2-GMEC) can selectively migrate into, accumulate, and express the transgene at metastatic sites of breast cancer (2, 3), and that the expression of rhIL-2 at the local sites the metastases resulted in significant reduction of the tumor burden (4). This report covers our investigations of: (a) the cellular mechanisms operational *in vivo* during hIL-2-GMEC-mediated regression of lung metastases from breast cancer, (b) whether microvascular endothelial cells can express IL-12 transgene, and secrete biologically active IL-12 protein, and (c) whether systemically-administered, genetically-modified endothelial cells (GMEC) can target sites of active and inactive physiological angiogenesis.

2. BODY

2.1 Specific Aims and Statement of Work

The specific aims of this research are (1) To determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites; (2) To determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases will induce an anti-tumor immune response. The approved Statement of Work is as follows:-

Task 1: **Months 1-24.**

Determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites.

a. Mouse lung endothelial cells (MLECs) will be isolated and enriched using FDG-FACS. The cells will be transduced with a retroviral vector containing human IL-2 gene.

b. Efficiency of IL-2/MLEC incorporation at different tumor sites:
   - Co-localization of IL-2/MLEC and tumor in animals: three experiments; 40 animals per experiment.

c. Determination of toxicity of IV IL-2/MLEC administration:
   - Acute toxicity following a single dose of 10^5 IL-2/MLEC administration
   - Cumulative toxicity following 3 IV injections of 10^5 IL-2/MLECs spaced 3-4 days apart.
   Three experiments; 40 animals per experiment.

d. Optimization of IL-2/MLEC incorporation in tumor sites:

-Tumor-bearing animals will receive three IV injections of IL-2/MLEC closely (3-4 days) or widely (5-7) apart. Expression of IL-2 transgene at the metastatic sites determined by RNA PCR amplification of human IL-2 in discrete individual metastases. Four experiments; 40-50 animals per experiment will be performed.
-Comparison of the relationship between different administration schedules with the number of cells incorporated at sites of tumor metastases will be determined. Two experiments; 40 animals per experiment will be performed.

Task 2: **Months 24-36.**

Determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases site will induce an anti-tumor immune response.

Groups of experimental of experimental and control animals will be sacrificed weekly to monitor hIL-2 expression in the lungs, quantitate metastases, and to assess lung tumor response to IL-2/MLEC treatment. One group of the experimental and control animals will be observed over time for survival. Survivors will receive additional MFP injection of 4T1 cells to determine their ability to reject tumor re-challenge.

2.2 Research Accomplishments

Overview

During the past year, we investigated the cellular mechanisms operational *in vivo* during hIL-2-GMEC-mediated regression of lung metastases from breast cancer, and whether systemically-administered, genetically-modified endothelial cells (GMEC) can target sites of active or inactive physiological angiogenesis. We also generated interleukin-12 (IL-12) gene-expressing endothelial cells. These cells secrete immunologically and biologically active IL-12 protein, and are currently being evaluated for ability to induce the regression breast cancer metastases, and confer a specific, long-term systemic immunity in mice.
To date, we have completed the experiments outlined in task 1 and most of the studies outlined in task 2.

MATERIALS AND METHODS

Animals

Female BALB/c mice, six to eight weeks of age, and purchased from the National Cancer Institute Animal Program (Frederick, MD) were used for this study. Protocols for husbandry and experimental manipulation were approved by the Georgetown University Animal Care and Use Committee. All mice were housed in a pathogen-free environment, and National Institutes of Health-Centers for Disease Control biosafety level 2 containment procedures were observed. This mouse strain was used these studies in order to ensure that the mice were syngeneic for both tumor cell lines and endothelial cells, thereby avoiding the immunological rejection of either tumor cell implants or the gene-modified endothelial cells.

Endothelial cell isolation

Mouse microvascular endothelial cells (MEC) were isolated from the lungs of BALB/c mice using a multi step method as previously reported (1). Endothelial cells obtained retained differentiated endothelial cell makers, including cobblestone morphology and expression of acetylated-low density lipoprotein (Ac-LDL) receptor and von Willebrand Factor. Endothelial cells at passages 3 or 4 were used for subsequent studies.

Generation of endothelial cells expressing neomycin resistance and human IL-2 or murine IL-12 trangenes

Endothelial cells expressing exogenous genes for neomycin phosphotransferace resistance (*Neo*^{R}) and human interleukin (hIL) 2 (hIL-2-GMEC) were generated (1). To generate endothelial cells expressing *Neo*^{R} and murine IL-12 trangenes (mIL-12-GMEC), a TFG-mIL-12-*neo* retroviral vector which expresses both p35 and p40 subunits of murine IL-12 as well as the *Neo*^{R} genes from a polycistronic message utilizing internal ribosome entry site sequences (5) was generously provided by Dr. Michael T. Lotze. Retroviral supernatant was generated by transfecting TFG-mIL-12-*Neo* proviral constructs into CRIP packaging cell line (5). The titer of the CRIP TFG-mIL-12 producer cell line used in the subsequent experiments was 5×10^4 CFU/ml. To generate rhIL-2-GMEC, microvascular endothelial cells (MEC) were harvested from lungs of BALB/c mice and transduced with helper-free, culture supernatants of the CRIP TFG-mIL-12 producer cell line. Twenty four hours prior to infection, MEC at passage 3 or 4 were seeded onto fibronectin-coated 100 mm plastic dishes at a 1:2-1:3 split ratio. Following overnight culture of the cells, the medium was replaced with 5 ml of amphotropic viral stock containing either the rmIL-2 gene (TFG-mIL-12-*Neo* construct) or the parent vector with only the neomycin resistance (*Neo*^{R}) gene (MFG-*Neo*) and 8μg/ml polybrene to promote viral attachment to cells. The dishes were returned to the incubator and an additional 5 ml of fresh endothelial cell growth medium was added after two to four hours. Subsequently, the dishes were incubated overnight and then refed with complete growth medium. Colonies were isolated by selection in G418 100μg/ml, the minimum concentration of G418 that was required to kill all cultured endothelial cells (data not shown). After selection in G418, cell populations were expanded and characterized for rmIL-12 production as well as maintenance of endothelial cell phenotype. Recombinant mIL-2 protein secretion *in vitro* by the rmIL-2-GMEC was assayed from 24-hour culture supernatant of the cells.

Our rhIL-2 + *Neo*^{R}*R*-, rmIL-12 + *Neo*^{R}- and *Neo*^{R} gene-expressing endothelial cells (rhIL-2-GMEC, rmIL-2-GMEC and *Neo*-GMEC, respectively) retained the endothelial cell phenotype, including cobblestone morphology, uptake of acetylated-low density lipoprotein (Ac-LDL), and expression of von Willebrand factor. No replication-competent helper virus was detected in the media of twenty-five passages in each of the gene-modified endothelial cells.

Quantitation of secreted rhIL-2 and rmIL-12 protein in endothelial cell culture media

Recombinant human interleukin 2 and murine interleukin 12 protein secreted by cytokine gene-transduced and non-transduced endothelial cells were assayed in the culture media of the cells by enzyme-linked immunoabsorbance (ELISA) assay (Quantikine human IL-2 and Quantikine M murine IL-12 p70, respectively) (QuantikineTM R&D Systems, MN) , following

the manufacturer's instructions. The range of sensitivity of the ELISA assays was 30-2000 pg/ml for IL-2, and 2.5-500 pg/ml for IL-12. Functional IL-2 activity was determined by a microtiter colorimetric assay using the tetrazolium salt, *2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide* (XTT), which is cleaved in mitochondria to a dark-blue formazan product (6), to measure the proliferation of HT-2 cells (ATCC; Rockville, MD) in response to the cytokine (7). The bioactivity of IL-12 produced *in vitro* by IL-12 gene transduced MMEC was determined by the proliferative response of day 4 PHA-activated lymphoblasts as previously described (5). IL-12 levels were expressed as nanograms/10^6/24 h. Recombinant hIL-2 secretion *in vitro* by the rhIL-2-GMEC ranged from 28-78 ng/10^6 cells/24h (i.e., 368-1000 IU/10^6 cells/24h) while the recombinant mIL-12 secretion *in vitro* by the rmIL-12-GMEC ranged from 32-85 ng/10^6 cells/24h

Establishment of pulmonary metastases of mammary adenocarcinoma

The 4T1 tumor cell line (provided by Fred R. Miller, Michigan Cancer Foundation), was derived from a spontaneously-arising mammary tumor in a BALB/c mouse. The cells were harvested from dishes while in their exponential growth phase in culture with 0.1% trypsin/EDTA, washed once with culture medium and twice with PBS. They were counted electronically using a Coulter counter (Coulter Counter, Model ZB, Coulter Corporation, FL, USA) and suspended in PBS at a concentration of 10^6 cells/ml. More than 98% of the cells in suspension were singlets and more than 90% were viable based on their ability to exclude trypan blue. To establish pulmonary metastases of 4T1 tumors, the 4T1 cell line was first implanted in syngeneic BALB/c mice. The primary tumors were established by injecting 1 X 10^5 4T1 cells suspended in 0.1ml of phosphate buffered saline into the first right thoracic mammary fat pad (MFP) of the mice. Two weeks following the inoculation of 4T1 cells into the MFP, when many pulmonary metastases are 1- 2 mm in diameter, i.e., a point at which they begin to acquire a new blood supply (8), the primary tumors were completely excised, and mice were randomized into three groups. One group of mice was treated with three sequential tail vein injections of 1 x 105 hIL-2-GMEC in 0.1 ml PBS. The second group received three sequential tail vein injections of 10^5 *Neo*-GMEC in 0.1 ml PBS, and the third group was given three sequential tail vein injections of 0.1 ml PBS without GMEC. The hIL-2-GMEC secreted 78 ng (i.e., 1000 IU) of rhIL-2 protein per 10^6 cells *in vitro* in 24 h. Administration of the hIL-2-GMEC was started on the third day following surgical excision of the primary tumor, and the injections were spaced 3 days apart. Three groups of non-tumor-bearing mice were also studied. These consisted of non-tumor-bearing mice given three sequential tail vein injections of 10^5 hIL-2-GMEC or *Neo*-GMEC, and non-tumor-bearing mice without GMEC. After GMEC administration, the animals were monitored for survival. Lungs of mice that died or sacrificed when they became ill were removed, processed and examined for number and size of pulmonary metastases. Otherwise, groups of mice were sacrificed at periodic intervals after the administration of the GMEC and various tissues, including individual lung metastasis were harvested and processed for hIL-2 gene expression using DNA polymerase chain reaction (PCR) and reverse transcriptase (RT-PCR) techniques, histology, and immunocytochemistry. Thereafter they were examined in order to follow the rate of growth or regression of the lung metastases, rhIL-2 gene expression, and the nature of cellular infiltrate of the tumors.

Histological and immunocytochemical analysis of explanted tumor metastases

Tissue from the site of tumor inoculation as well as individual metastatic foci in the lungs were excised, fixed in 10% formal-saline and embeded in paraffin wax, sectioned at 4 μm., and stained with Haematoxylon- and eosin. For immunocytochemistry, tissues were embeded in OCT compound (Miles Laboratory, Elkert, IN), snap-frozen in liquid nitrogen, and preserved at -80oC until sectioning. 5-μm cryostat sections were fixed in acetone and immunostained with purified rat mAb to: CD45 (MI/9.3.4 HL2 hybridoma, T200), CD8 (53.6.72 hybridoma, Lyt 2), CD4 (GK1.5 hybridoma, L3T4), anti-MAC-1 (MI/70.15.11.HL hybridoma), MAC-3 (M37/84.6.34 hybridoma:ATCC) and mouse granulocyte (RB6-8C5 hybridoma: Pharmigen, San Diego, CA). These were pre-incubated with rabbit serum to prevent nonspecific binding, and sequentially incubated with the optimal dilutions of the various mAb for 1 h, a rabbit anti-rat IgG (Pharmigen, San Diego, CA) for 30 min, and a rat peroxidase antiperoxidase (Abbot Laboratories, North Chicago, IL) for 1 h. Each incubation step was followed by a 5-min Hanks balanced salt solution (HBSS) wash. The sections were finally incubated with 0.03% hydrogen peroxide (H_2O_2) and 0.06% 3.3' -diaminobenzidine (Sigma Chemicals Co St. Louis, MO) for 3 to 5 min. The slides were then washed for 5 min in running tap water, counter stained with Haematoxylon for 1 min, and mounted in Canadian balsam. Endogenous peroxidase was inhibited by pretreatment with 0.01% v/v H_2O_2. Neutrophils did not show the inhibition-resistant endogenous peroxidase activity typical of eosinophils. Control slides were obtained by omitting the mAb to control the rabbit ant-rat Ig cross-reactivity, and using rat ant-human primary antibodies of the same IgG subclass. The percentage of immunostained cells over total cells was determined at 400 x magnification on five microscope fields with the help of a 1-mm square grid. Neutrophils were characterized by segmented or doughnut nuclei. Also, the presence of the typical oval granule with central rectangular crystalloid in the cytoplasm of the eosinophils allowed them to be unmistakably distinguished from neutrophils. Quantitative and semi-quantitative determination of the cellular infiltrate in the individual tumor specimen were performed. Haematoxylon- and eosin-stained sections from frozen tissue blocks were examined and the number of lymphocyte, granulocytes and macrophages per high-power fields scored.

Physiological Angiogenesis Model

Wound Healing was used as a model of physiological angiogenesis for our studies. Mice were anaesthetized with methoxyflurane and the flank skin was shaved and cleansed with 70% ethanol. A 5-mm full-thickness wound was then made by pinching up a fold of the flank skin and incising through the two layers of the skin using a new, sterile disposable blade (Baker Cummins Dermatological). The incision was closed with 3-0 sutures. Two to four days after wound surgery, a time when maximum angiogenic activity is observed at the site of the wound, the mice were randomized into three groups and given various treatments. One group was given a single dose of 10^5 *LacZ* gene-modified mouse microvascular endothelial cells (*lacZ*-GMEC) by tail vein injection. The second group received three three sequential tail vein injections of 10^5 *lacZ*-GMEC in 0.1 ml PBS, a dose found to be safe and effective in our previously published studies (2). The third group was given three sequential tail vein injections of 0.1 ml PBS without *LacZ*-GMEC. to serve as the GMEC treatment control. A group of normal female mice without surgery

received three sequential tail vein injections of 10^5 *lacZ*-GMEC in 0.1 ml PBS to serve as the angiogenesis control. Administration of the *LacZ*-GMEC was started on the third day following surgical excision of the primary tumor, and the injections were spaced 3 days apart. Seven days after the administration of *lacZ*-GMEC, a time when maximum accumulation of IV-administered gene modified endothelial cells is observed at site of angiogenesis (2,10), the animals were euthanized. Immediately thereafter, various tissue samples, including the skin from the wound site with a perimeter of 2- 4 mm of normal skin tissue, ovary, mammary glands, and uteri were obtained from the animals, processed for histology, *Griffonia simplicifolia agglutinin* (GSA) lectin staining or PECAM-1 (CD31) immunohistochemistry, and β-galactosidase histochemistry. Thereafter, the tissues were examined for the presence and amount of *lacZ*-expressing endothelial cells.

Tissue fixation and X-gal Immunohistochemical staining

Various tissues harvested from individual animals were processed separately for β-galactosidase histochemistry and histology. The organs were first washed in PBS, pH 7.4 and then fixed in a solution of phosphate-buffered saline (PBS) containing 2% (vol/vol) formaldehyde and 0.2% (vol/vol) glutaraldehyde for 60 minutes. After fixation, tissues were rinsed three times in PBS and incubated for 24 hours at 4°C in X-gal staining solution (X-gal 1 mg/ml; 5 mM potassium ferricyanide; 5 mM potassium ferrocyanide; 2 mM $MgCl_2$; 0.02% [vol/vol] Nonidet P-40; 0.01% [wt/vol] sodium deoxycholate in PBS). At the end of the incubation period, organs were rinsed with 3% dimethyl sulfoxide in PBS followed by PBS alone. The organs were then visualized and photographed using an Olympus SZH stereo microscope. Subsequently, X-gal-stained tissues were fixed further at 4°C for 2 hours in 2% paraformaldehyde in 0.1M PBS, pH 7.4, followed by rinsing in 0.1M PBS and dehydration in 3% sucrose in 0.1M PBS prior to performing cryosections.

Subsequently, tissue sections were counter-stained with Harris Haematoxylon, washed in distilled water, dehydrated in sequential baths of ethanol and xylene, dried, mounted and examined with a Zeiss Axiphot microscope. Photomicrographs were taken under the Zeiss Axiphot microscope with Kodak Ektar 25 film (ASA 100). In tissue staining experiments, sections were also processed for Haematoxylon and eosin staining to obtain descriptive histology.

Analysis of X-gal-stained tissues for *LacZ*-GMEC targeting *in vivo*

After X-gal staining, tissues from experimental and control animals were examined macroscopically with the help of an Olympus SO stereo (dissecting) microscope fitted with a fiber optic illuminator. Thereafter, both X-gal-positive and negative angiogenic sites within individual tissues were carefully examined with a dissecting microscope before photograph. Targeting of the angiogenic sites by *LacZ*-expressing cells was determined by the presence and amount of positive X-gal staining within and/or around the sites. The relative tissue X-gal staining was scored on a scale of negative (-); + for few positive single cells; ++ for few positive cell clusters; +++ for moderate number of positive cell clusters; ++++ for widespread positive of cell clusters as judged by visual inspection.

RESULTS

Expression of recombinant human interleukin 2 (rhIL-2) and Cellular infiltrates at local tumor sites

To determine the potential immune effector cells that are involved in hIL-2/GMEC-mediated regression of lung metastases from a mammary adenocarcinoma, we analyzed individual lung metastasis for rhIL-2 gene expression and cellular infiltrate. All tumors obtained from untreated, *Neo*-GMEC- or PBS-treated mice were negative for hIL-2 gene expression, whereas 2-80% of the tumors obtained from rhIL-2-GMEC-treated, tumor-bearing mice were positive for rhIL-2 gene expression by RT-PCR. The results of the histological and immunocytochemical analyses of these metastases are summarized in Table 1. There was an infiltration of non-specific inflammatory cells in most tumors that were negative for hIL-2 gene expression. The cells consisted of granulocytes and macrophages, and were confined to the edges of the tumors. In contrast, hIL-2 gene expression-positive tumors had an extensive infiltration of both specific and non-specific immune effector cells that was made up of granulocytes (mainly neutrophils), macrophages, CD4+ and CD8+ lymphocytes. More importantly, these cellular infiltrate were present at the periphery and center of the rhIL-2-positive tumors, indicating an ability of these cells to infiltrate the entire tumor mass.

Microvascular endothelial cell expression of mIL-12 transgene

The level of IL-12 secretion by various transduced cells are shown in Table 2. Cultured murine microvascular endothelial cells (MMEC) expressed recombinant mIL-12 gene in a stable fashion in vitro. All MMEC infected with TFG-mIL-12-*Neo* secreted significant amounts of rmIL-12 protein *in vitro*. MMEC transduced with the parent MFG-*Neo* vector exhibited a pattern of mIL-12 expression that was similar to the parent (non-transduced) cells. Another interesting observation was that the rmIL-12 produced was both immunologically and biologically active, suggesting that splicing events between IRES domains did not occur, and that endothelial cells could produce and post-translationally process both subunits of IL-12. Furthermore, rmIL-12-expressing MMEC retained endothelial cell differentiated marker, including cobblestone morphology and expression of acetylated-low density lipoprotein (Ac-LDL) receptor and von Willebrand Factor, suggesting that endothelial cell expression of exogenous IL-12 gene may not effect the expression of their endogenous genes.

Incorporation of genetically modified endothelial cells at sites of wound healing and inactive angiogenesis.

In our previous study, we demonstrated that IV-administered genetically-modified endothelial cells (GMEC) migrated and preferentially integrated into blood vessels at sites of angiogenesis that were induced by either acidic fibroblast growth factor (aFGF)-secreting cells (9) or tumor cells (2). To determine whether circulating GMEC can target sites of active or inactive physiological angiogenesis, we investigated the ability of IV-injected GMEC to

selectively migrate into, survive, and express exogenous gene(s) at sites of wound healing and old (healed) wounds.

The results of these experiments are summarized in Table 3. All the tissues with little or no active angiogenesis (normal skin, scar, ovary, mammary glands, and uteri) obtained from *lacZ*-GMEC-treated mice were negative for X-gal staining. In contrast, X-gal positive cells were seen in the immediate vicinity of wound healing in the skin from *lacZ*-GMEC-treated mice. Moreover, there was an increase in the intensity of X-gal-positive staining (i.e. *lacZ*-GMEC incorporation) at these sites following the administration of three sequential IV injections of 10^5 *lacZ*-GMEC. We also observed that the intensity of X-gal staining seen at the sites of wound healing decreased as angiogenesis (determined by *Griffonia simplicifolia agglutinin* (GSA) lectin or PECAM-1 [CD31] immunohistochemical staining) subsided with time, suggesting a direct relationship between the level of angiogenesis and GMEC incorporation at these sites. The specificity of the X-gal-staining due to *lacZ*-GMEC targeting was confirmed by the absence of X-gal-positive cells at sites of active angiogenesis due to wound healing in the skin from mice treated with PBS or three IV injections of *Neo*-GMEC (endothelial cells transduced with Neo^R gene-containing vector (Table 3).

DISCUSSION

In our previous reports, we demonstrated ability of circulating hIL-2-GMEC to accumulate and express rhIL-2 at the local sites of breast cancer metastases (4, 10) or melanoma (2) *in vivo*. The presence of rhIL-2 protein at these sites was associated with a significant reduction of the established metastases. We have also shown that both macrovascular and microvascular endothelial cells expressing human IL-2 transgene can induce potent NK cell and CTL activation (1). In the present study, we observed that expression of recombinant hIL-2 gene at the local sites of lung metastases of breast cancer induced an increased infiltration of neutrophils, macrophages, and both CD4+ and CD8+ lymphocytes. The presence of these cells and their distribution within and around the tumor mass suggest that they may be involved in the rhIL-2/GMEC-mediated inhibition of the growth of breast cancer *in vivo* (4, 10). This is supported by reports in the literature of similar findings during IL-2-mediated regression of a variety tumors *in vivo* (11-18), and the ability of IL-2 to stimulate and activate these immune effector cells (1, 18-20).

Although encouraging, the results of our present and previous studies indicate that single IL-2 gene therapy may have a limited benefit in the treatment of advanced breast cancer. This is supported by the reports that transduction and expression of human or murine IL-2 cDNA by a variety of weakly or non-immunogenic tumor cell lines delays the formation of the tumors, but did not induce long-term protective immunity against the tumors in animals (21-26). A synergistic action between IL-2 and/or other genes with anti-tumor effects may provide a more feasible option for clinical application. To explore this possibility, we initiated studies to determine the ability of endothelial cells to express murine IL-12 transgene. This cytokine has been shown to induce complete rejection of a variety of tumors, and to confer a specific, long-term systemic immunity in animals with advanced tumors (27-30). To date, we have successfully generated IL-12 transgene-expressing endothelial cell clones (mIL-12-GMEC) that produce high

amounts rmIL-12 *in vitro* (Table 2). Experiments to determine the therapeutic efficacy of the mIL-12-GMEC in mice bearing lung metastases of breast cancer are in progress.

The detection of *lacZ*-expressing GMEC in the immediate vicinity of wound healing suggest that angiogenic factors elaborated by physiological processes can facilitate the targeting, survival and activities of circulating genetically-modified endothelial cells (GMEC) at sites of active physiological angiogenesis. Our observation that the intensity of X-gal staining (*lacZ*-GMEC incorporation) decreases as angiogenesis wanes, and the absence of *lacZ*-GMEC in tissues with little or no active angiogenesis indicate that the presence and activities of circulating GMEC at a specific location is dependent upon the degree of angiogenesis at that location. Clearly, the implications of these findings in terms of the clinical benefits and safety of systemic administration of GMEC will have to be further explored in future studies.

2.3 Plans for the Future

In the coming year, we plan to complete all the ongoing experiments, including the determination of the ability of microvascular endothelial cells (MECs) expressing interleukin-12 (IL-12) transgene gene to inhibit the growth of established breast cancer metastases in mice.

3. Key Research Accomplishments

To date, we have:

1) Isolated pure population of lung endothelial cells from BALB/c mice. The cells have been transduced with a retroviral vector containing human IL-2 gene and high expressing clones have been isolated and fully characterized.
b) Determined the efficiency of hIL-2/MLEC incorporation into sites of breast cancer metastasis.
c) Optimized hIL-2/MLEC incorporation into sites of pulmonary metastasis of breast cancer.
d) Determined acute and cumulative toxicity of IV-administered hIL-2/MLECs.
e) Established that the expression of hIL-2 transgene at the local site of pulmonary metastases site can induce an anti-tumor immune response *in vivo*.
f) Determined the level the nature, level, and duration of anti-tumor immune response that is induced at the local tumor site following IV administration of hIL-2/MLEC.
g) Determined the ability of IV-administered, genetically-modified endothelial cells to target sites of active and inactive physiological angiogenesis.
h) Generated interleukin (IL) 12 transgene-expressing endothelial cells and their therapeutic utility are currently being determined in a mouse mammary adenocarcinoma model.
i) Published a manuscript of some of our study results. Another manuscript is in preparation.
j) Obtained an NIH-RO1 grant award based on the results of these studies.

4. Reportable Outcomes

A) Manuscripts:

1. Endothelial cell-based systemic gene therapy of metastatic melanoma. Ojeifo JO, Lee HR, Rezza P, Su N, Zwiebel JA. Cancer Gene Therapy. 8(9): 2001 (In press).

2. Ojeifo JO, Notario V, Herscowtiz HB, Zwiebel JA. Interleukin-2 gene-modified endothelial cell therapy of metastatic breast cancer in a murine model (In preparation)

B) Grant awards:

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Grant title: Combined Gene Therapy for Metastatic Breast Cancer.
PI: John O. Ojeifo
Duration: 01/01/02 – 12/31/06

5. Conclusions

The results of our present studies demonstrate that: (1) both non-specific and specific immune effector cells composed of granulocytes (mainly neutrophils), macrophages, CD4+ and CD8+ lymphocytes, may be involved in hIL-2-GMEC-mediated inhibition of the growth of metastatic breast cancer cells *in vivo*, (2) fully differentiated microvascular endothelial cells can stably express exogenous IL-12 gene, and secrete immunologically and biologically active rmIL-12 *in vitro,* without affecting the expression of the endogenous genes, and (3) circulating *lacZ* gene-expressing endothelial cells (*lacZ*-GMEC) can migrate into and accumulate in the vicinity of active physiological angiogenesis.

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Appendices:

(a) Letter of grant award from National Cancer Institute, NIH.
(b) Letter of acceptance of manuscript entitled "Endothelial cell-based systemic gene therapy of metastatic melanoma" for publication in *Cancer Gene Therapy*.

Table 1: Cell composition of the reactive infiltrate in lung metastases of mammary adenocarcinoma obtained from untreated and hIL-2/GMEC-treated, tumor-bearing mice .

Type of Reactive Leukocyte[a]	Level of reactive leukocytes in 4T1 tumor Metastases	
	hIL-2-negative Metastases	hIL-2-positive Metastases
$CD45^+$	Moderate, Scattered positive	Extensive, diffusely positive
Pan T (Thy 1)	Negative	Moderate to extensive , scattered positive
$CD4^{+b}$	Negative	Moderate, scattered positive
$CD8^+$	Negative	Low-moderate, occasional- diffusely positive
Neutrophils	Moderate, scattered positive	Extensive, diffusely positive
Eosinophils	Low, scattered positive	Low, scattered positive
Macrophages	Low, occasional scattered positive	Extensive, diffusely positive

[a]Determined semi-quantitatively by examining cells in five sites of the same tumor area. Cells were stained with specific mAbs as described in *Materials and Methods*. Cell counting to determinate the number of the specific immune effector cell infiltrate in each experimental group of tumors is in progress.

[B]Besides $CD4^+$ subset of T lymphocytes, the GK1.5and anti-CD4 mAb used stains macrophages.

Table 2: Recombinant murine interleukin 12 (rmIL-12) production by parental and rmIL-12 gene-transduced mouse microvascular endothelial cells (MMEC)

Cell	Populations		IL-12 (ng/10^6 cells/24h)[a]
MMEC	Polyclonal		0
Neo-MMEC	Polyclonal		0
IL-12-MMEC	Polyclonal		40.4
IL-12-MMEC	Oligoclonal #		
		1	32.1
		2	44.1
		3	58.6
		4	74.2
		5	85.2

[a]The bioactivity of IL-12 produced in vitro by IL-12 gene transduced MMEC was determined by the proliferative response of day 4 PHA-activated lymphoblasts as previously described (5). IL-12 levels were expressed as nanograms/10^6/24 h.

Table 3: Incorporation of *LacZ* gene-expressing endothelial cells (*LacZ*-GMEC) at sites of active and inactive physiological angiogenesis in mice following IV administration of the *LacZ*-GMEC.

Tissue	GMEC Implant	Presence and Amount of X-gal staining[a] (i.e. *lacZ*-GMEC incorporated)
Normal Skin	*LacZ*-GMEC	-[b]
Ovary	*LacZ*-GMEC	-
Mammary glands	*LacZ*-GMEC	-
Uterus	*LacZ*-GMEC	-
Skin with wound Healing	*LacZ*-GMEC (single IV dose of 10^5 cells)	+
Skin with wound Healing	*LacZ*-GMEC (Three IV doses of 10^5 cells)	++
Skin with wound Healing	*Neo*-GMEC	-
Skin with wound Healing	PBS	-
Scar	*LacZ*-GMEC	-

[a]Determined by careful visual inspection of X-gal stained tissues using an Olympus SO stereo (dissecting) microscope fitted with a fiber optic illuminator as described in *Materials and Methods*

[b]The relative tissue X-gal staining was scored on a scale of negative (-); + for few positive single cells; ++ for few positive cell clusters; +++ for moderate number of positive cell clusters; ++++ for widespread positive of cell clusters as judged by visual inspection.

Appendices

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

JOHN O OJEIFO
GEORGETOWN UNIV/MED CTR
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3970 RESERVOIR ROAD, NW
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Date : 08-AUG-01

RE: 1R01CA093495-01

DEAR DR. OJEIFO:

Congratulations! On behalf of the National Cancer Institute (NCI), I am pleased to inform you that your application was rated in the peer review process as having significant and substantial scientific merit. The purpose of this letter is to provide you with early notification that the NCI has selected your grant application, as referenced above, for funding in FY 2002. Under separate cover, NCI program staff will send you a copy of the summary statement prepared by the Scientific Review Administrator of the Initial Review Group that evaluated your application.

It is our goal to expedite the processing of your application. At this time we cannot commit to either an exact dollar amount or an actual start date of the award. Both will depend upon further NCI staff review of the application and summary statement concerns as well as other issues such as Other Support and applicable program reductions directed by NCI funding plan decisions.

If you have any general questions regarding this expedited award process, please call Ms. Barbara Lamb, Manager, Awards, Records and Control Center, on 301-496-7756.

We wish you every success with your research.

Sincerely .

Leo F. Buscher Jr.
Chief Grants Management Officer
National Cancer Institute

cc : Business Official
Suresh Arya

GRANTS & CONTRACTS OFFICER
GEORGETOWN UNIVERSITY
RES & TECHNOLOGY DEVLOPMENT SER
4000 RESERVOIR RD NW177 BLDG,D
WASHINGTON, DC 20007

Date: 06/22/01
To: Dr. Ojeifo:
Subj: Grant Number 1R01CA93495-01
Program Code 2Q
From: LEO BUSCHER
Chief, Grants Operations Branch

Application 1R01CA93495-01 entitled:
COMBINED GENE THERAPY FOR METASTATIC BREAST CANCER
has received a priority score of 221 and a percentile of 16.1.

This application and other applications will be forwarded to National Advisory Council or Board for review. This is not a notice of a grant award: funding decisions will not be made until after the Council or Board makes recommendations.

Please submit the following documentation to the National Institutes of Health office identified below.

- Updated Other Support - For all Key Personnel, provide details on how you would adjust any overlapping other support if this application is funded. Please use PHS Form 398, page 7-GG.

- If Human Subjects are involved in your proposed research, please provide the OHRP Assurance type and number, the Certification of Institutional Review Board Approval, and certification that each individual identified under "key personnel" in the proposed research has completed an educational program in the protection of human subjects. Refer to:
http://grants.nih.gov/grants/guide/notice-files/NOT-OD-00-039.html and
http://grants.nih.gov/grants/news.htm for futher information.

The documentation identified above and a copy of this letter should be submitted with a cover letter countersigned by an authorized institutional business official as soon as possible to the following office:

LEO BUSCHER
Chief Grants Operation Branch
NIH, NATIONAL CANCER INSTITUTE
EPS/234
BETHESDA, MD 20892-7148
Phone Number: 301-496-7753
Fax Number: 301-402-3409
Email: LB45U@NIH.GOV

A Summary Statement, containing evaluative comments and budget recommendations, will be sent to you in approximately eight weeks. After receiving your Summary Statement, you may call the program administrator listed below to discuss the contents. Should a revised application be indicated, follow the instructions in the application kit and respond specifically to the comments in the Summary Statement. Current NIH policy limits the number of amended versions of an application to two, and that these must be submitted within two years of the date of the original version of the application.

Submission of the above information will enable us to expedite the issuance of an award should this application be identified for funding. If you have any additional questions, please contact:

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Chief, Grants Operations Branch, LEO BUSCHER

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MS #0[illegible]-597[R1]

May 24, 2001

James A. Zwiebel, M.D.
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6130 Executive Blvd
Rockville, MD 20852

Dear Dr. Zwiebel:

The ~~revised version of your manuscript~~ entitled, "Endothelial Cells-based Systemic Gene Therapy of Metastatic Melanoma" has been accepted for publication in *CANCER GENE THERAPY*. You will receive galley proofs directly from the publisher.

Once again, thank you for submitting your work to *CANCER GENE THERAPY*.

Very truly yours,

Robert E. Sobol, M.D.

RES/ls

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Endothelial cell–based systemic gene therapy of metastatic melanoma

John O. Ojeifo,[1] Hyeran R. Lee,[1] Phyllis Rezza,[2] Ning Su,[1] and James A. Zwiebel[3]

Departments of [1]Oncology and [2]Pathology, Georgetown University Medical Center and Vincent T. Lombardi Cancer Center, N.W. Washington, District of Columbia 20007; and [3]Biologics Evaluation Section, Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institute of Health.

Cancer metastasis accounts for a significant proportion of morbidity and mortality in patients. Effective means of treating disseminated disease remains elusive. The purpose of this study was to determine whether genetically modified endothelial cells (GMEC) can selectively target and deliver recombinant therapeutic molecules to sites of tumor metastases. Following the establishment of lung metastases of 4T1 mammary tumor in mice, intravenously (i.v.) administered, *lacZ* transgene–expressing endothelial cells (*lacZ*-GMEC) accumulated at the tumor sites. An average of 32% and 90% of the pulmonary metastases were X-gal stained following one and three tail vein injections of 10^5 *lacZ*-GMEC, respectively. The linear pattern of X-gal staining seen within the tumor sites and the histological appearance of the tumor vasculature were consistent with the incorporation of *lacZ*-GMEC into blood vessels. In C57Bl/6 mice harboring lung metastases of melanoma, the administration of three sequential i.v. injections of 10^5 endothelial cells expressing a human interleukin 2 transgene abrogated the tumor metastases and prolonged survival of the animals. These results demonstrate that i.v.-administered GMEC can selectively accumulate, survive, and stably express exogenous genes at multiple tumor sites. These findings support a role for i.v.-administered GMEC as a potential therapeutic strategy for the systemic treatment of cancer metastases. **Cancer Gene Therapy (2001) 8**

Key words: Interleukin 2; endothelial cells; gene therapy; murine melanoma.

Although there have been substantial advances in early detection and treatment of human cancers, survival for patients with certain forms of cancer has not improved.[1–3] The prognosis for advanced solid epithelial tumors remains dismal, in part due to the occurrence of metastatic disease in many patients at the time of diagnosis,[3,4] and the ineffectiveness of currently available methods to treat metastatic disease. Consequently, morbidity and/or mortality remain high in this subset of patients, and novel treatment strategies are needed.

The growth and metastasis of tumors are dependent on the formation of neo-vessels that arise in response to angiogenic factors elaborated by tumor cells themselves.[5–10] In previous reports, we and others have shown that the process of angiogenesis can be exploited to deliver recombinant molecules to the sites of newly forming blood vessels by using genetically modified endothelial cells (GMEC). In particular, when *lacZ* gene–transduced endothelial cells (*lacZ*-GMEC) were injected intravenously, they migrated into and preferentially accumulated at sites of active angiogenesis induced by fibroblast growth factor (aFGF)–secreting NIH 3T3 cells.[11] Also, locally injected *lacZ*-GMEC can integrate into blood vessels formed in response to ischemia[12] or brain tumors.[13,14] These findings suggest that physiologically driven or tumor-initiated angiogenesis could be used to promote the delivery of recombinant molecules to the angiogenic sites.

The delivery of therapeutic molecules that would interfere with tumor growth and metastases or their vascular supply may now be feasible using implants of genetically modified cells.[14–17] Endothelial cells are excellent vehicles for drug delivery because of their ability to express a wide variety of endogenous[18] and exogenous genes,[17,19–21] and their strategic location within the vascular compartment. Initially, we and others found that injection of cytokine[22] or prodrug gene–expressing[20] endothelial cells *directly* into tumor sites resulted in significant antitumor effects. Because sites of metastases are areas of active angiogenesis,[23,24] and i.v.-injected GMEC can selectively migrate into these sites,[11] we decided to investigate whether *systemic* administration of GMEC may be a useful approach for targeting recombinant therapeutic molecules to metastatic sites throughout the body. To determine the feasibility of an endothelial cell–based systemic gene therapy for metastatic cancer, we investigated[1] the efficiency of multifocal tumor targeting by i.v.-administered, *lacZ*-GMEC, and[2] the therapeutic potential of i.v.-injected, recombinant human interleukin 2 (rhIL-2) transgene–expressing endothelial cells in a murine experimental metastasis model. Our studies dem-

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onstrate efficient targeting and eradication of tumor metastases by the systemic administration of GMEC.

MATERIALS AND METHODS

Animals

Female BALB/c and C57Bl/6 mice, 6–8 weeks of age, were purchased from the National Cancer Institute Animal Program (Frederick, MD) and used for this study. Protocols for husbandry and experimental manipulation were approved by the Georgetown University Animal Care and Use Committee. All mice were housed in a pathogen-free environment, and National Institutes of Health-Centers for Disease Control biosafety level 2 containment procedures were observed. Experiments to determine the efficiency of GMEC targeting of multiple tumor sites were conducted on female BALB/c mice whereas a C57Bl/6 mouse model was used to evaluate the ability of cytokine gene–modified endothelial cells to target multiple tumor sites and induce tumor regression. The different strains of mice were used for the respective studies to ensure that the mice were syngeneic for both tumor cell lines and endothelial cells, thereby avoiding the immunological rejection of either tumor cell implants or the gene-modified endothelial cells.

Endothelial cell isolation

Mouse microvascular endothelial cells (MECs) were isolated from the lungs of BALB/c and C57Bl/6 mice using a multistep method as previously reported.[19] Briefly, lungs were removed from the animals and minced into 1–2-mm pieces, washed, and digested with collagenase (Worthington Biochemical, Freehold, NJ; 2 mg/mL in phosphate-buffered saline [PBS]), for 20 minutes at 37°C in a shaker water bath at 80 cycles/min. Subsequently, the products of digestion were transferred to a sterile 50-mL disposable conical tube containing isolation medium (medium 199 [M-199; Biofluids, Rockville, MD] buffered with 0.71 g sodium bicarbonate [$NaHCO_3$]/L and 2.21 g HEPES/L; and supplemented with 0.1% bovine serum albumin [BSA] and 25 mg soybean trypsin inhibitor [STI], pH 7.4) and allowed to pellet at unit gravity for 30 minutes at 4°C. The supernatant was passed through a 110-μm pore size nylon screen (Nitex, Tetko, Elmsford, NY) to remove the large fragments. The filtrate was passed through a 20-μm pore size nylon screen that was washed three times with isolation medium. Capillary and tissue fragments retained on the 20-μm screen were collected, then washed with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solutions (CMF-HBSS), pH 7.4, and suspended in 14 mL of CMF-HBSS buffered with 0.35 g $NaHCO_3$/L and supplemented with 0.25% BSA and 25 mg STI/L, pH 7.4. Subsequently, the cell suspension was thoroughly mixed with 21 mL iso-osmotic Percoll with 10× CMF-HBSS and centrifuged in a fixed angled rotor (JA-20, Beckman) at 20.000×*g* for 60 minutes at 4°C. Tufts of capillary endothelial cells from the third- to the tenth-milliliter fractions from the top of the gradient (density, 1.006–1.050 g/mL) were collected, washed, and seeded on fibronectin-coated dishes containing endothelial cell growth medium (M-199 with 20% fetal calf serum, 2 mM glutamine, 50–100 μg/mL endothelial cell growth supplement, and 50 U/mL heparin). After a 72-hour incubation period in a humidified incubator of 95% air and 5% CO_2 maintained at 37°C, the contaminating non-endothelial cells were removed by labeling the cells with DiI-acetylated low-density lipoprotein (DiI-Ac-LDL, Biomedical Technologies, Stoughton, MA) and sorting with the fluorescence-activated cell sorter (FACS) as described by Voyta et al.[25] Endothelial cells obtained were examined for maintenance of differentiated endothelial cell makers, including cobblestone morphology and expression of acetylated low-density lipoprotein (Ac-LDL) receptor[25] and von Willebrand factor.[26]

Confluent monolayer of the cells was examined under low power of a phase-contrast microscope for cobblestone morphology. Cells evaluated for expression of Ac-LDL receptor were cultured to semiconfluence. Thereafter, the cells were incubated with 10 μg/mL of DiI-Ac-LDL (Biomedical Technologies) in endothelial cell growth medium for 4 hours at 37°C. At the end of the incubation period, culture medium was removed and the cells were washed twice with probe-free medium for 5 minutes. The cells were examined with standard rhodamine excitation/emission filters. More than 95% of the sorted cells retained these differentiated endothelial cell makers. Endothelial cells at passages 3 or 4 were used for retroviral transduction.

Construction of retroviral vectors containing lacZ gene or rhIL-2 gene and transduction of endothelial cells

Retroviral vectors containing *lacZ* or rhIL-2 gene were constructed as previously reported.[11,19]

Culture supernatants containing infectious IL-2–containing retroviral particles are produced by introducing the retroviral vector plasmids into the PA317 amphotropic packaging cell line.[27] To generate *lacZ* gene–modified endothelial cells (*lacZ*-GMEC), the viral supernatant from the packaging cell line was used to transduce murine MECs that were isolated from the lungs of BALB/c mice. Twenty four hours before infection, MECs at passage 3 or 4 were seeded onto fibronectin-coated 100-mm plastic dishes at a 1:2–1:3 split ratio. Following overnight culture of the cells, the medium was replaced with 5 mL of amphotropic viral stock with a titer of 5×10^5 colony-forming units per milliliter titer and containing either the *lacZ* gene vector construct (LZSN) or the parent vector with the neomycin resistance (neo^R) gene alone (LXSN) and 8 μg/mL of polybrene. After 2–4 hours incubation of the cells with the viral medium, 5 mL of virus-free, endothelial cell growth medium containing 8 μg/mL of polybrene was added to each dish and incubated overnight at 37°C. Thereafter, the cells were cultured in endothelial cell growth medium without polybrene. At confluence, the cells were sorted using FDG-FACS. Following two sequential cell sorts, more than 95% of the *lacZ*-GMEC expressed the product of *lacZ* gene, β-galactosidase, as demonstrated by 4-Cl-5-Br-3-indolyl-β-D-galactopyranoside (X-Gal) staining. Both *lacZ*-GMEC and neo^R gene–expressing endothelial cells (*neo*-GMEC) retained the endothelial cell phenotype, including cobblestone morphology, uptake of Ac-LDL,[25] and expression of von Willebrand factor.[26] No replication-competent helper

virus was detected in the culture medium of *lacZ*-GMEC or *neo*-GMEC during 25 passages of these cells.

To generate rhIL-2-GMEC, MECs were harvested from lungs of C57Bl/6 mice and transduced with retroviral vector containing hIL-2 gene under the transcriptional control of cytomegalovirus promoter as previously reported.[19] Briefly, helper-free culture supernatant from PA317 amphotropic packaging cell clones producing ~10^6 G418-resistant colony-forming units per milliliter was used to infect the endothelial cells. Twenty four hours before infection, MECs at passage 3 or 4 were seeded onto fibronectin-coated 100-mm plastic dishes at a 1:2–1:3 split ratio. Following overnight culture of the cells, the medium was replaced with 5 mL of amphotropic viral stock containing either the rhIL-2 gene (LNCIL-2 construct[19]) or the parent vector with only the neo^R gene (LNCX construct) and 8 μg/mL polybrene to promote viral attachment to cells. The dishes were returned to the incubator and an additional 5 mL of fresh endothelial cell growth medium was added after 2–4 hours. Subsequently, the dishes were incubated overnight and then refed with complete growth medium. Colonies were isolated by selection in 100 μg/mL G418, the minimum concentration of G418 that was required to kill all cultured endothelial cells (data not shown). After selection in G418, cell populations were expanded and characterized for rhIL-2 production as well as maintenance of endothelial cell phenotype. Recombinant hIL-2 secretion *in vitro* by the rhIL-2-GMEC was assayed from 24-hour culture supernatant of the cells.

The *lacZ*-, rhIL-2–, and neo gene–expressing endothelial cells (*lacZ*-GMEC, rhIL-2-GMEC, and *neo*-GMEC, respectively) retained the endothelial cell phenotype, including cobblestone morphology, uptake of Ac-LDL, and expression of von Willebrand factor. Again, no replication-competent helper virus was detected in the media of 25 passages in each of the gene-modified endothelial cells.

Quantitation of secreted rhIL-2 in culture media

rhIL-2 protein secreted by cytokine gene–transduced and nontransduced endothelial cells were assayed in the culture media of the cells by enzyme-linked immunoabsorbance assay (ELISA; Quantikine RD Systems, MN), following the manufacturer's instructions. The range of sensitivity of the ELISA assays was 30–2000 pg/mL for IL-2. Functional IL-2 activity was determined by a microtiter colorimetric assay using the tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylaminocarbonyl]-2*H*-tetrazolium hydroxide (XTT), which is cleaved in mitochondria to a dark-blue formazan product,[28] to measure the proliferation of HT-2 cells (ATCC, Rockville, MD) in response to the cytokine.[29] Recombinant hIL-2 secretion *in vitro* by the rhIL-2-GMEC ranged from 28 to 78 ng/10^6 cells per 24 hours (i.e., 368–1000 IU/10^6 cells per 24 hours) (Table 1).

Tumor cell lines. The 4T1 tumor cell line (provided by Fred R. Miller, Michigan Cancer Foundation), was derived from a single, spontaneously arising mammary tumor in a BALB/c mouse. B16F10 melanoma cell line was obtained from ATCC. The cell lines were grown in DMEM supplemented 10% fetal calf serum and 2 mM glutamine. The cells were harvested from dishes while in their exponential growth phase in culture with 0.1% trypsin/EDTA, washed once with culture medium and twice with PBS. They were counted electronically using a Coulter counter (Model ZB, Coulter, Miami, FL) and suspended in PBS at a concentration of 10^6 cells/mL. More than 98% of the cells in suspension were singlets and more than 90% were viable based on their ability to exclude trypan blue.

In vivo *tumor models*

Establishment of pulmonary metastases and administration of lacZ-GMEC. To determine the efficiency of multifocal tumor targeting by i.v.-administered *lacZ*-GMEC, a metastatic murine mammary tumor cell line, 4T1, was implanted in syngeneic BALB/c mice. The primary tumors were established by injecting 1×10^5 4T1 cells suspended in 0.1 mL PBS into the first right thoracic mammary fat pad (MFP) of the mice. Ten days after inoculation of 4T1 cells, when pulmonary metastases were first observed, mice were injected with *lacZ*-GMEC *via* the tail vein. Control groups included 4T1 tumor–bearing mice without GMEC or injected with 10^5 *neo*-GMEC, and non–tumor-bearing mice with or without *lacZ*-GMEC treatment. Tumor-bearing animals that received *lacZ*-GMEC were injected with either 10^5, 10^6, or 10^7 *lacZ*-GMEC *via* the lateral tail vein. At intervals of 4, 7, and 14 days after i.v. GMEC administration, animals in each group were sacrificed to follow the fate of injected cells over time. The primary tumor, skin, lungs, liver, spleen, bones, brain, and heart were removed and processed for histology and β-galactosidase histochemistry to visualize the *lacZ*-expressing endothelial cells.

To determine the effect of multiple inoculations of relatively small doses of GMEC on the efficiency of tumor targeting, mice bearing 4TI lung metastases were given three sequential tail vein injections of 10^5 *lacZ*-GMEC. In this set of experiments, 2 weeks following the inoculation of 4T1 cells into the MFP, when many pulmonary metastases are 1–

Table 1. Recombinant Human IL-2 (rhIL-2) Production by Parental and rhIL-2 Gene–Transduced Mouse Microvascular Endothelial Cells (MECs) *in Vitro*

Cell	Population	IL-2 (ng/10^6 cells per 24 hours)
MEC	Polyclonal	0
neo-GMEC	Polyclonal	0
rhIL-2-GMEC	Polyclonal	2–8 (26–105 IU)*
rhIL-2-GMEC	Oligoclonal no.	
	1	28 (368 IU)
	2	44 (578 IU)
	3	40 (526 IU)
	4	76 (1000 IU)
	5	58 (763 IU)
	6	46 (605 IU)

*76 pg of IL-2=1 International unit (IU, also known as W.H.O. unit). See R&D Systems — WHO conversion table at the Internet: http://www.rndsystens.com/asp/c_who_add.asp).

2 mm in diameter, i.e., a point at which they begin to acquire a new blood supply,[24] the primary tumors were excised and mice were randomized into three groups. One group of mice was given three sequential tail vein injections of 10^5 *lacZ*-GMEC in 0.1 mL PBS. The second group received three sequential tail vein injections of 10^5 *neo*-GMEC in 0.1 mL PBS, and the third group was given three sequential tail vein injections of 0.1 mL PBS without GMEC. Administration of the GMEC was started on the third day following surgical excision of the primary tumor, and the injections were spaced 3 days apart. Three groups of non–tumor-bearing mice were also studied. These consisted of non–tumor-bearing mice given three sequential tail vein injections of 10^5 *lacZ*-GMEC or *neo*-GMEC, and non–tumor-bearing mice without GMEC. At weekly intervals, mice from each group were sacrificed to follow the fate of injected cells over time. Various tissues were excised from the animals and processed for histology and β-galactosidase histochemistry as described below.

rhIL-2-GMEC administration to mice bearing B16F10 melanoma pulmonary metastases. We employed an experimental metastasis model to evaluate the therapeutic potential of endothelial cell–based gene therapy approach for metastatic cancer. Pulmonary metastases were established by tail vein injection of 1×10^5 B16F10 melanoma cells into C57Bl/6 mice. Seven to ten days following injection (when lung tumors ranged from 0.5–2 mm in diameter), the mice were given the first of three intravenous injections of 1×10^5 mouse MECs expressing rhIL-2 (rhIL-2-GMEC), spaced 3 days apart. *In vitro* IL-2 secretion by rhIL-2-GMEC ranged from 28 to 78 ng/10^6 cells per 24 hours, i.e., 368 to 1000 IU/10^6 cells per 24 hours (Table 1). The control groups consisted of (1) mice that received the tumor cells and three injections of *neo*-GMEC, (2) mice that received the tumor cells alone, (3) mice that received three injections of rhIL-2-GMEC alone, and (4) mice that received neither tumor cells nor GMEC. Purified rhIL-2 protein was not administered to animals in these studies because toxic doses of rhIL-2 would have been required to attain the serum levels of 2.8–7.8 ng (36.8–100 IU) of rhIL-2 that would be comparable to the levels produced by the rhIL-2-GMEC at tumor sites.[30,31] After GMEC administration, the animals were monitored for survival. Lungs of mice that died or were sacrificed when they became ill were removed and examined for number and size of pulmonary metastases. Otherwise, groups of mice were sacrificed at periodic intervals after the administration of the GMEC and their lungs examined to follow the rate of regression of the lung metastases.

Tissue fixation, X-gal and CD31 immunohistochemical staining

Various tissues, including lungs, brain, liver, spleen, bone, skin, and heart harvested from individual animals were processed separately for β-galactosidase histochemistry and histology. The organs were first washed in PBS, pH 7.4, and then fixed in a solution of PBS containing 2% (vol/vol) formaldehyde and 0.2% (vol/vol) glutaraldehyde for 60 minutes. After fixation, tissues were rinsed three times in PBS and incubated for 24 hours at 4°C in X-gal staining solution (X-gal 1 mg/mL; 5 mM potassium ferricyanide; 5 mM potassium ferrocyanide; 2 mM $MgCl_2$; 0.02% [vol/vol] Nonidet P-40; 0.01% [wt/vol] sodium deoxycholate in PBS). At the end of the incubation period, organs were rinsed with 3% dimethyl sulfoxide in PBS followed by PBS alone. The organs were then visualized and photographed using an Olympus SZH stereo microscope. Subsequently, X-gal–stained tissues were fixed further at 4°C for 2 hours in 2% paraformaldehyde in 0.1 M PBS, pH 7.4, followed by rinsing in 0.1 M PBS and dehydration in 3% sucrose in 0.1 M PBS before performing cryosections.

In addition to β-galactosidase histochemistry, CD31 immunohistochemical staining of the excised tissues was performed by means of a double antibody-labeling method. X-gal–stained tissues were fixed in neutral buffer containing 3.7% formalin for >24 hours, processed, and embedded in paraffin. The paraffin tissue blocks were cut into 5-μm sections, placed on glass slides, and deparaffinized in sequential baths of xylene and 100% ethanol. After rehydration in PBS and treatment with 0.1% trypsin in PBS at 37°C for 30 minutes, the tissue sections were blocked for endogenous peroxidase activity by incubation in 0.3% H_2O_2–absolute methanol for 30 minutes at room temperature. This pretreatment was followed by three washes in PBS, blocking for nonspecific staining with 2% normal rabbit serum and 5% BSA, and incubation in primary antibody solution (5 μg/mL purified rat anti-mouse CD31 [Pharmingen] in blocking solution) for 2 hours at room temperature. Following three washes in PBS, tissue sections were covered with 50 μL of biotinylated rabbit anti–rat IgG (Vector Laboratories, Burlingame, CA) at 5 μg/mL dilutions in blocking solution. After a 60-minute incubation at room temperature, slides were washed again in PBS, processed for peroxidase staining using a commercial avidin–biotin complex method kit (Vectastain Elite ABC kit; Vector Laboratories) and developed with 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB) substrate (metal enhanced DAB substrate kit; Pierce). Subsequently, tissue sections were counterstained with Harris hematoxylin, washed in distilled water, dehydrated in sequential baths of ethanol and xylene, dried, mounted, and examined with a Zeiss Axiphot microscope. Photomicrographs were taken under the Zeiss Axiphot microscope with Kodak Ektar 25 film (ASA 100). In tissue-staining experiments, sections were also processed for hematoxylin and eosin staining to obtain descriptive histology.

Analysis of tissues for lacZ-GMEC targeting in vivo

After X-gal staining, various tissues from experimental and control animals were examined macroscopically with the help of an Olympus SO stereo (dissecting) microscope fitted with a fiber optic illuminator. Lungs were first separated into individual lobes. Thereafter, X-gal–positive and –negative tumor nodules on the surface of the lobes were counted using the dissecting microscope before photograph. Targeting of the tumors by *lacZ*-expressing cells was determined by the presence and amount of positive X-gal staining within and/or around the tumor sites. The relative tissue X-gal staining was scored on a scale of

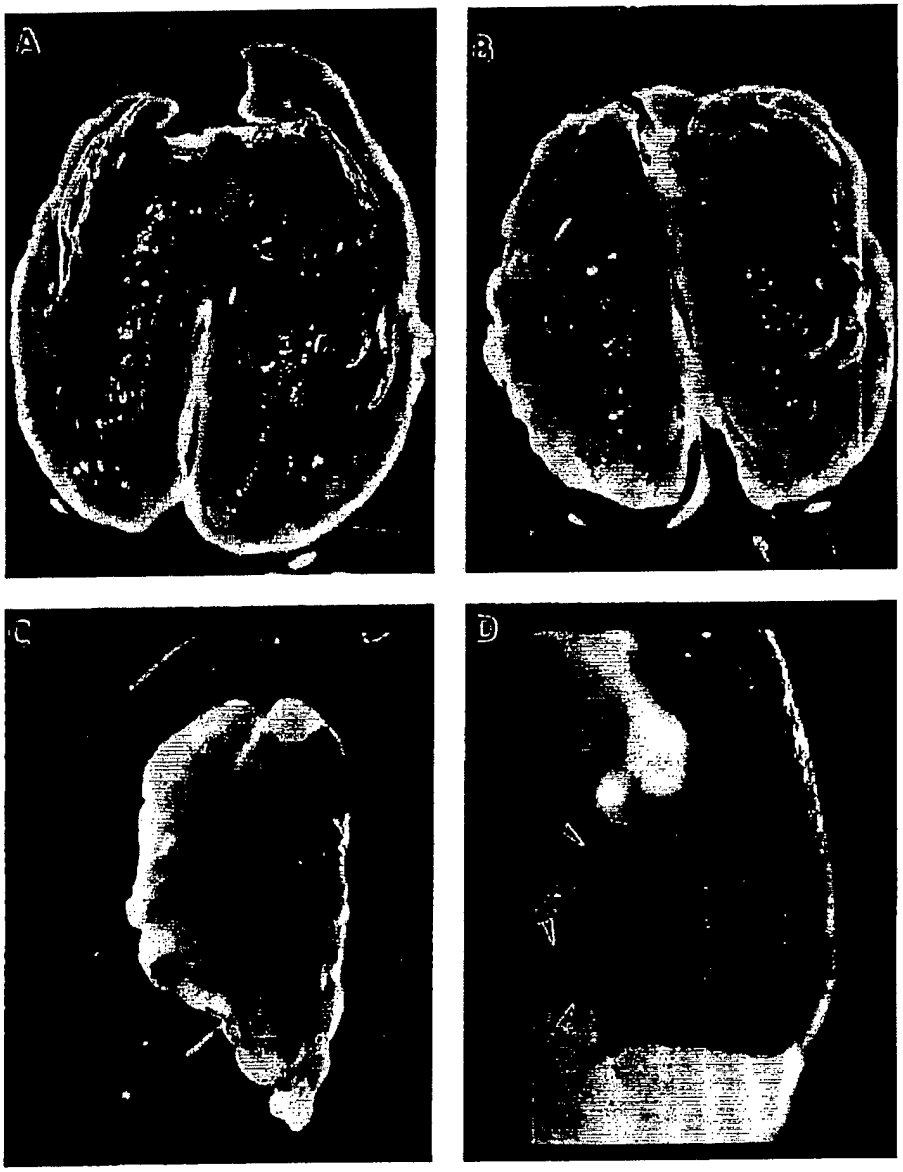

Figure 1. *In vivo* targeting of 4T1 tumor metastases with genetically modified endothelial cells (GMEC). **A:** X-gal–stained lungs of a mouse 4 weeks following MFP inoculation with 10^5 4T1 mammary carcinoma cells. **B:** X-gal–stained lungs of 4T1 tumor-bearing mouse 2 weeks following a single tail vein injection of 10^5 *neo*-GMEC (mouse lung endothelial cells transduced with the parent vector lacking the *lacZ* gene). Note the absence of X-gal staining in both lungs. **C** and **D:** Lungs of 4T1 tumor–bearing mice 2 weeks following a single intravenous injection of 10^5 *lacZ*-GMEC. Note the presence of X-gal–positive (blue) cells in or around pulmonary metastases (**C**, arrows) and X-gal–positive branching structures radiating from an X-gal–positive metastatic deposit (**D**, arrowheads). Magnification: **(A)** ×35; **(B)** ×32; **(C)** ×43; **(D)** ×197.

negative (−); + for few positive single cells; ++ for few positive cell clusters; +++ for moderate number of positive cell clusters; ++++ for widespread positive of cell clusters, as judged by visual inspection.

Table 2. Tissue Distribution of *lacZ*-GMEC in 4T1 Tumor-Bearing and Non–Tumor-Bearing BALB/c Mice Following a Single Tail Vein Injection of 10^5, 10^6, or 10^7 *lacZ* Gene–Modified Endothelial Cells (*lacZ*-GMEC)

		X-Gal–positive organs (presence and amount of X-gal staining)							
								Subcutaneous tissue	
Treatment	Duration of GMEC implant or time from GMEC administration (d)	Brain	Heart	Lung (mets)	Liver	Bone	Spleen	Tumor sites	Othersite
i.v. *lacZ*-GMEC alone	14	0/5*	0/5	0/5	0/5	0/5	0/5	NA	Na
4T1+neo^R-GMEC	14	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4T1+*lacZ*-GMEC	14								
(a) 10^5		0/5	0/5	5†/5	0/5	0/5	0/5	5‡/5	0/5
(b) 10^6		0/5	0/5	3†/5	0/5	0/5	0/5	3‡/5	0/5
(c) 10^7		0/5	0/5	2†/5	0/5	0/5	0/5	2‡/5	0/5

NA, not applicable; *mets*, metastases. *Results expressed as number of X-gal–positive mice/total number of mice injected. †Moderate to widespread X-gal–positive cell clusters. ‡Indicates few X-gal–positive cells.

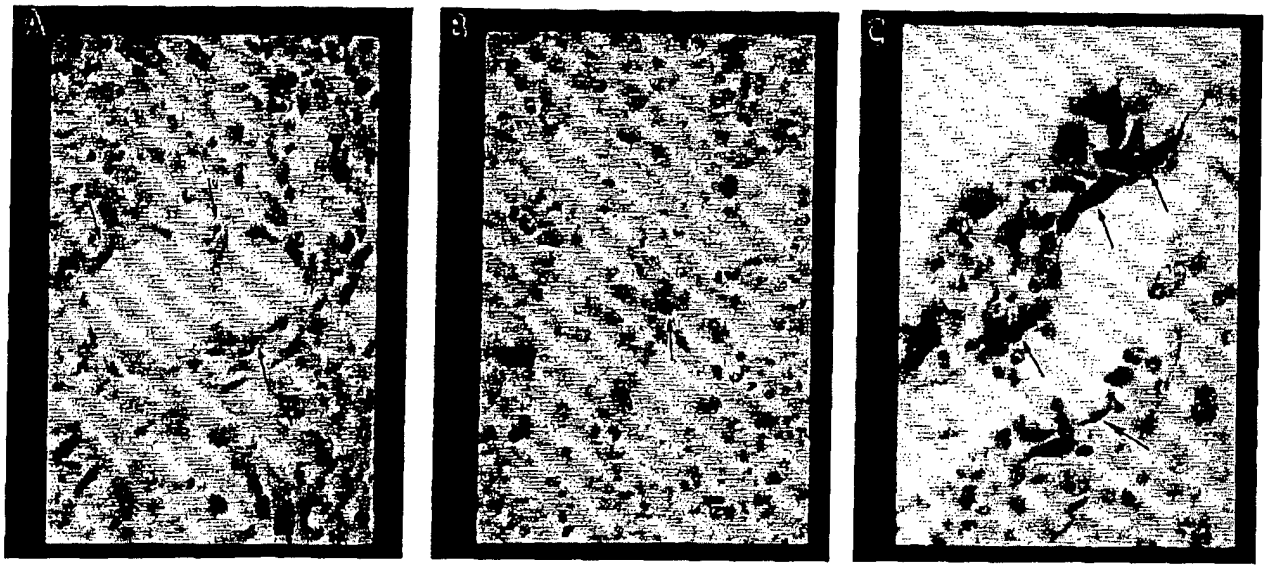

Figure 2. Histological sections of mouse lungs 4 weeks following MFP inoculation with 10^5 4T1 cells, and 2 weeks following tail vein injection with 10^5 *lacZ*-expressing syngeneic endothelial cells. The lungs were stained either with X-gal alone (**A** and **B**) or both X-gal and PECAM-1 [CD31] antibody immunohistochemical stains©). **A:** X-gal–positive *lacZ*-GMEC (blue cells [arrows]) participating in capillary lumen formation. Note presence of blood elements in the lumen. ×658. **B:** X-gal–positive *lacZ*-GMEC (implanted endothelial cell [blue cells]) undergoing mitosis. ×658. **C:** Longitudinal section of a blood vessel showing PECAM-1– (yellowish-brown color) and X-gal (blue color)–positive cells (arrows) alongside their PECAM-1–positive but X-gal–negative host counterparts. Magnification: ×329.

Survival analysis of B16/F10 melanoma-bearing mice treated with i.v. injections of rhIL-2-GMEC

Survival studies were set up with different treatment groups of animals in an identical manner. Study endpoints were death or sacrifice of the animal when they appeared to be in distress, as evidenced by lethargy, difficulty in breathing, refusal to feed, or ruffled hair. Generally, untreated tumor-bearing animals started to exhibit some of these symptoms between 18 and 21 days after i.v. inoculation of B16/F10 melanoma cells. All animal study protocols were approved by the Georgetown University Animal Care and Use Committee. Ethical guidelines for conducting research on laboratory animals were followed throughout these studies.

Statistical analysis

The Student's t test was used to compare the results between the control and experimental groups. A P value of less than .05 was considered to be statistically significant.

RESULTS

Targeting of pulmonary metastasis of breast tumors by lacZ transgene–expressing endothelial cells

In our previous study, we found that i.v.-administered GMEC migrated and preferentially integrated into blood vessels at sites of active angiogenesis that were induced by acidic fibroblast growth factor (aFGF)-secreting cells.[11] To determine whether macrometastases of breast tumors can sustain a level of angiogenesis that will enable migrating GMEC to survive, proliferate, and take part in neoangiogenesis at the local site, we administered a single i.v. injection of 10^5 *lacZ*-GMEC to mice with lung metastases of 4T1 mammary carcinoma. Two weeks following the administration of *lacZ*-GMEC to the tumor-bearing ani-

Table 3. *In Vivo* Targeting of Tumor Metastases With *lacZ* Gene–Modified Endothelial Cells (*lacZ*-GMEC): Percentage of X-gal–Positive Tumor Metastases Following a Single Tail Vein Injection of 10^5, 10^6, or 10^7 *lacZ*-GMEC in BALB/c mice

Study group	Treatment (no. of mice)*	No. of metastases per mouse	Dose and type of GMEC administered	% of metastases with X-gal–positive cells (mean±SD)
1	4T1 Tumor+*lacZ*-GMEC (5)	>200	10^5 *lacZ*-GMEC	31.9±12.9
2	4T1 Tumor+*lacZ*-GMEC (5)	>200	10^6 *lacZ*-GMEC	10.7±5.1
3	4T1 Tumor+*lacZ*-GMEC (5)	>200	10^7 *lacZ*-GMEC	2.5±0.7
4	4T1 Tumor+*neo*-GMEC (5)	>200	10^5 *neo*-GMEC	0
5	4T1 Tumor alone (5)	>200	0	0
6	*lacZ*-GMEC alone (5)	0	10^5 *lacZ*-GMEC	0
7	None (5)	0	–	0

*Numbers in parentheses indicate total number of mice treated.

Table 4. *In Vivo* Targeting of Tumor Metastases with *lacZ* Gene–Modified Endothelial Cells (*lacZ*-GMEC): Percentage of X-gal–Positive Tumor Metastases Following Three Sequential Tail Vein Injections of 10^5 *lacZ*-GMEC in BALB/c Mice

Study group	Treatment (no. of mice)*	No. metastases per mouse (mean±SD)	% of metastases with X-gal–positive cells (mean±SD)
1	4T1 Tumor+*lacZ*-GMEC (13)	110±27	93.5±3
2	4T1 Tumor+*Neo*-GMEC (8)	108±35	0
3	4T1 Tumor alone (13)	111±32	0
4	*lacZ*-GMEC alone (8)	0	0
5	None (5)	0	0

*See for explanation of symbols.Table 3

mals, X-gal staining was observed at the sites of pulmonary metastases (Fig 1C). The X-gal–positive cells were seen in the immediate vicinity of the metastatic foci in the lungs. Small numbers of X-gal–stained cells were also detected at the subcutaneous site of the primary tumor. No X-gal–stained cell was seen in other tissues of these animals. Also, no X-gal–positive cell was seen in the lungs of untreated, tumor-bearing mice (Fig 1A), or tumor-bearing mice given *neo*-GMEC (endothelial cells transduced with the parent vector lacking the *lacZ* gene construct) (Fig 1B). Furthermore, we did not find X-gal–positive cells in *lacZ*-GMEC–treated, non–tumor-bearing mice (Table 2), consistent with the hypothesis that tumor-associated angiogenesis promoted the survival of *lacZ*-GMEC at tumor sites. Interestingly, some *lacZ* gene–expressing cells were seen forming luminal structures containing blood elements, including leukocytes and erythrocytes (Fig 2A), consistent with the incorporation of *lacZ*-GMEC within blood vessels. Other *lacZ* gene–expressing cells were seen undergoing mitosis (Fig 2B). The endothelial cell origin of the *lacZ* gene–expressing cells was confirmed using anti–PECAM-1 (CD31) immunohistochemistry (Fig 2C).

Efficiency of GMEC targeting of tumor metastases in vivo

Once we had established that GMEC preferentially become incorporated within neo-vessels at metastatic sites, we investigated the efficiency of *lacZ*-GMEC tumor targeting. Two weeks after a single systemic administration of 10^5 *lacZ*-GMEC cells, we observed that about 32% of 4T1 lung

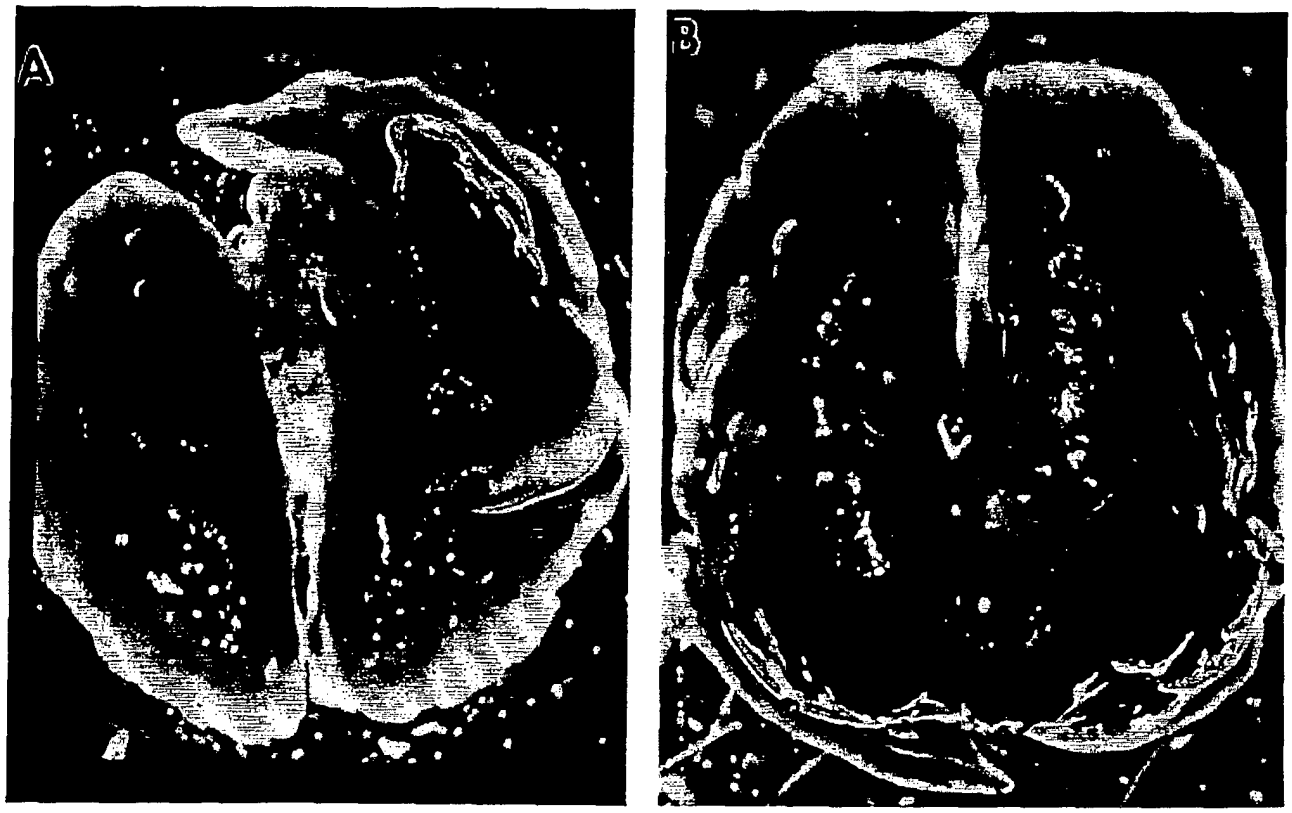

Figure 3. *In vivo* targeting of 4T1 tumor metastases with *lacZ*-GMEC. X-gal–stained lungs of 4T1 tumor-bearing mice 1 **(A)** or 2 weeks **(B)** following three sequential tail vein injections, one injection given every 3 days, of 10^5 *lacZ*-expressing syngeneic endothelial cells. Note X-gal–positive staining of almost the entire lungs in **(B)**. Magnification: **(A)** ×30, and **(B)** ×32.

metastases became X-gal positive (Table 3). The pattern of X-gal staining of the metastatic foci ranged from a few clusters of blue staining cells to diffuse blue staining of the tumors. Some tumors exhibited minimal or spotty staining (Fig 1C), whereas others were diffusely X-gal positive (Fig 1, C and D). The diffusely stained foci comprised about 10% of the individual metastases. Attempts to improve the targeting efficiency by increasing the dose of *lacZ*-GMEC up to 10^7 cells were unsuccessful. Whereas all the five mice treated with a single injection of 10^5 *lacZ*-GMEC had X-gal staining in and around their lung tumors, only three and two of the five mice in the groups that received 10^6 *and* 10^7 *lacZ*-GMEC, respectively, had detectable X-gal staining. Also, only focal punctate staining or a few X-gal–positive cell clusters were seen in these animals (Table 2). The remaining animals in each group became ill and subsequently died following the administration of the larger doses of GMEC. These deaths may have occurred due to vascular occlusion in the lungs due to the large inoculum of GMEC.

To increase the targeting efficiency of *lacZ*-GMEC, we modified our protocol as follows: Once pulmonary metastases reached 1–2 mm in diameter, the primary tumor was removed to avoid continuous tumor seeding of the lungs. Three i.v. doses of 10^5 *lacZ*-GMEC were then administered to each animal, spaced 72 hours apart. The multiple *lacZ*-GMEC injections markedly increased the GMEC-targeting efficiency to multi focal tumor sites (Table 4 and Figure 3) without any observable ill effects in the animals. Two weeks after the third i.v. dose of *lacZ*-GMEC, more than 94% of the individual lung metastases in tumor-bearing mice contained X-gal–positive cells (Fig 3 and Table 4). Furthermore, the percentage of individual metastases with widespread *lacZ*-positive cells increased to more than 60% (Fig 3). The specificity of the X-gal staining due to *lacZ*-GMEC targeting in these mice was again confirmed by the absence of X-gal–positive cells in the lungs of mice that received *lacZ*-GMEC alone, tumor cell alone, or tumor cells plus endothelial cells transduced with neo^R gene–containing vector (*neo*-GMEC) (Fig 1, A and B; Table 2).

Effects of rhIL-2-GMEC on the growth of established pulmonary metastases of B16/F10 melanoma

Having established that multiple i.v. administration of GMEC can effectively target tumor metastases with little or no toxicity in animals, we evaluated the therapeutic utility of this strategy in an experimental metastasis model of murine melanoma. We have previously shown that both macrovascular and microvascular endothelial cells expressing a human IL-2 transgene induce potent NK cell and CTL activation.[19] Also, rhIL-2 expression within and around tumors can elicit an antitumor effect.[22,32,33] Based on these observations, we predicted that the production of rhIL-2 by rhIL-2-GMEC in the tumor microenvironment would result in the activation of effector cells while avoiding the toxicities that are associated with systemic cytokine administration.[32] To test this hypothesis, 1×10^5 B16F10 melanoma cells were administered by tail vein injection to immunocompetent, syngeneic, C57Bl/6 mice. Seven to ten days following tail vein injection of these cells, at a time when many lung tumors became "established" (i.e., tumors ranged from 1 to 2 mm in diameter), we administered three i.v. injections of the rhIL-2-GMEC *via* tail vein, spaced 72 hours apart. This multiple injection schedule was based on our earlier studies with the 4T1 mouse tumor model described above. The rhIL-2 produced by GMEC that were injected into animals ranged from 28 to 78 ng/10^6 cells per 24 hours (368–1000 IU/10^6 cells per 24 hours) (Table 1). As shown in Figure 4, the systemic administration of rhIL-2-GMEC caused a significant reduction in the tumor burden in the animals when compared to untreated or *neo*-GMEC–treated B16F10 tumor–bearing mice. Histological examinations

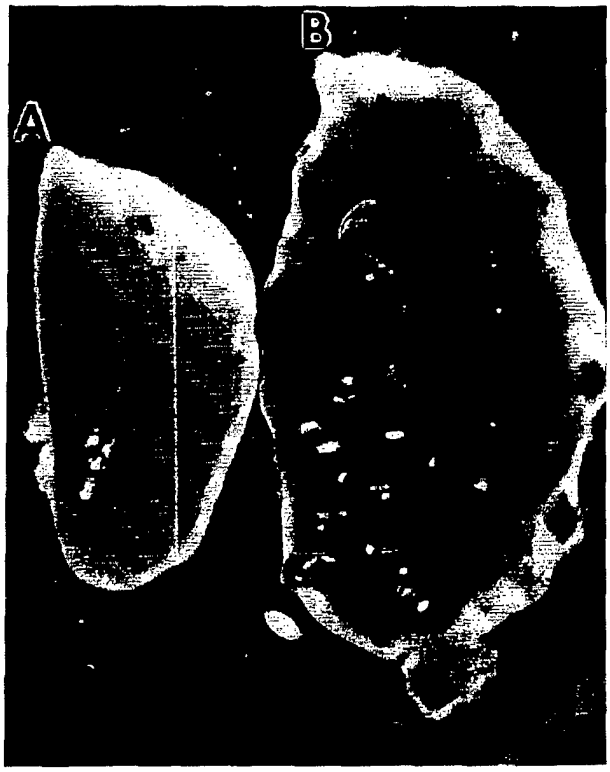

Figure 4. Macroscopic appearance of lungs from B16F10 melanoma tumor–bearing C57Bl/6 mice treated with rhIL-2 transgene–expressing endothelial cells **(A)** and untreated (controls, **B**). A total of 1×10^5 B16F10 melanoma cells were injected into C57Bl/6 mice (eight or more per group) *via* the tail vein. Seven to 10 days later, the animals received the first of three intravenous injections of 1×10^5 rhIL-2-GMEC or *neo*-GMEC spaced 3 days apart. The animals were then monitored for survival. The lungs of mice that died or were sacrificed when they became ill were removed and examined for the number and size of pulmonary metastases. To follow the rate of regression of lung metastases, other mice were sacrificed at periodic intervals after GMEC administration, their lungs removed and examined under a dissecting microscope. Note the presence of very few melanin pigment-containing metastatic foci in the lung from a treated mouse **(A)**, whereas the lung from an untreated mouse **(B)** contains numerous metastatic foci. Magnification: ×31.

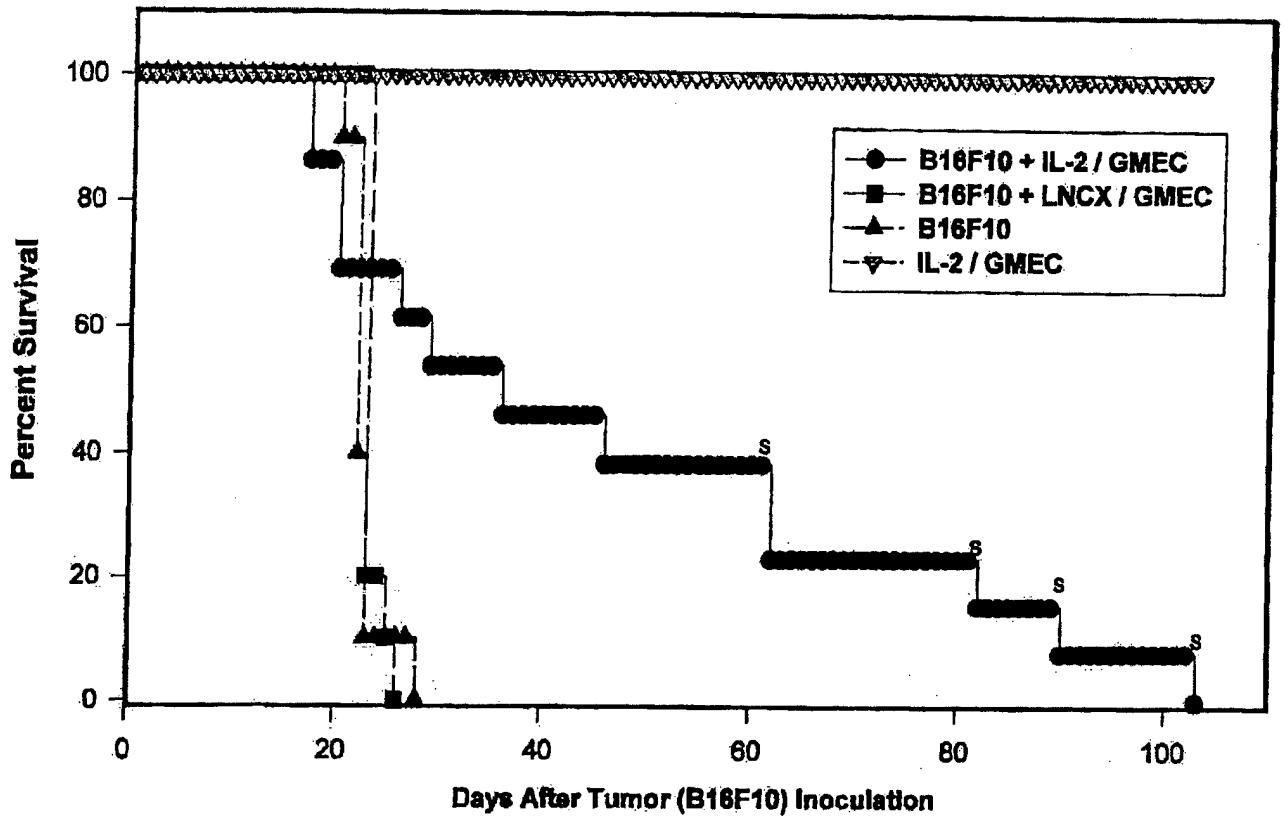

Figure 5. Survival of B16F10 melanoma tumor–bearing C57Bl/6 mice treated with rhIL-2 transgene–expressing endothelial cells. C57Bl/6 mice (eight or more per group) were injected with 1×10^5 B16F10 melanoma cells from *in vitro* culture. Seven to 10 days later, the mice were given the first of three intravenous injections of 1×10^5 rhIL-2-GMEC spaced 72 hours apart. The median survival times after the injection of B16F10 tumor cells: untreated, 23 days; vector (*neo*-GMEC)–treated, 24 days; rhIL-2-GMEC–treated, 42 days ($P<.02$); rhIL-2-GMEC–treated non–tumor-bearing mice, no deaths. Arrow heads indicate time points of rhIL-2-GMEC injection. "S" indicate animals that were sacrificed to assess effect of rhIL-2-GMEC on the growth of B16F10 lung tumors.

of the lungs from untreated or *neo*-GMEC–treated, tumor-bearing mice revealed numerous, large, melanin pigment–containing tumor foci. In contrast, the lungs of tumor-bearing mice treated with rhIL-2-GMEC contained very few, small tumor foci (Fig 4).

All control tumor-bearing animals, i.e., untreated tumor-bearing mice or tumor-bearing mice treated with *neo*-GMEC, died by day 28. The median survival of untreated and *neo*-GMEC–treated tumor-bearing mice following the injection of B16F10 cells were 23 and 24 days, respectively. In contrast, B16F10-bearing mice treated with rhIL-2-GMEC exhibited prolonged survival with a median of 42 days ($P<.02$) (Fig 5). Most of the deaths of rhIL-2-GMEC–treated animals resulted from elective sacrifice to follow the rate of regression of the lung metastases and not due to illness. Thus, the reduction in tumor burdens was corroborated by an increase in survival indicating a marked antitumor effect by rhIL-2-GMEC treatment. Animals that received rhIL-2-GMEC alone without tumor cells remained alive and well (Fig 5), indicating that rhIL-2-GMEC administration was well tolerated. Furthermore, we did not find any significant statistical difference in the number of metastatic foci seen in the lungs of tumor-bearing mice with or without a GMEC implant, nor did we observe the appearance of tumors in normal mice that received multiple doses of rhIL-2-GMEC alone, suggesting that systemic administration of the gene-modified endothelial cells did not promote tumor development in the animals.

DISCUSSION

Immune surveillance is compromised in patients with cancer and in tumor-bearing animals.[34–38] A promising biological approach to the therapy of cancer metastases involves the use of cytokines to enhance the body's immune defenses against tumors.[32] IL-2, arguably the best characterized molecule used in this regard, will activate certain cells derived from the large granular lymphocyte population, causing those cells to exhibit antitumor cytolytic activity.[32,33] IL-2 may also directly activate $p56^{lck}$, a protein associated with the IL-2 receptor β-chain, thereby decreasing the susceptibility of $CD8^+$ cells to inactivation by signals delivered by tumor cells.[38] Although a number of clinical trials have been carried out utilizing IL-2 or IL-2–activated lymphocytes, this promising approach has been compromised by the severe dose-limiting toxicities associated with the systemic administration of IL-2.[32]

As a more effective method of achieving high intratumoral concentrations of cytokines and of minimizing their toxicity, various gene therapy approaches, including (a) local delivery of cytokine genes to tumors by means of retroviral or adenoviral vectors, liposomes, or as naked DNA,[39–41] (b) treatment using loco-regional delivery of gene-modified primary endothelial cells,[20,22] or (c) cancer vaccines consisting of tumor cells[32,42–45] engineered to express IL-2 constitutively and/or other immune-activating genes in a paracrine manner, have been tested. Compared with systemically delivered cytokine proteins, these forms of immunotherapies have been shown to reduce tumorigenicity and metastasis in animal models without serious adverse effects.[20,22,39–42] However, still lacking is a means of delivering therapeutic genes to metastatic sites throughout the body. A number of promising tumor or tumor vasculature targeting approaches are being studied, including the modification of the retroviral envelope glycoprotein, the use of antibodies, tumor/tissue-specific promoters, endothelial cell–specific receptors, and angiogenesis-specific peptides.[46–54] Another novel form of targeted drug delivery system (our model) involves the systemic administration of primary endothelial cells that have been engineered to express therapeutic gene product(s) and that can target angiogenic sites.[11] The present study was designed to determine whether such a delivery method was feasible and effective for established metastases.

Because angiogenesis is an essential step in the growth and metastasis of tumors,[23,24] we reasoned that the process of tumor angiogenesis might enable circulating GMEC to integrate into the developing capillary network of a tumor and subsequently express recombinant genes.[11–13,15,16,20] To test this hypothesis, we initially administered a single dose of syngeneic, *lacZ*-expressing endothelial cells into animals bearing lung metastases. As predicted, i.v.-administered GMEC migrated into and preferentially accumulated at the sites of tumor metastases in the lungs. X-gal–positive cells were also seen at the subcutaneous sites of the primary tumor, suggesting that i.v.-injected GMEC can also migrate into tumor sites outside the lungs (Table 2). Apparently, in response to angiogenic factors elaborated by tumor cells at these places, GMEC were able to survive, proliferate, and become incorporated into the newly forming capillary networks (made up of both endogenous endothelial cells and GMEC) (Fig 2A–C). Furthermore, both vessel-associated and proliferating GMEC maintained endogenous (PECAM-1) and recombinant (*lacZ*) gene expression at the tumor sites (Fig 2A–C). These results extend our earlier observations,[11] and suggest that angiogenic factors produced by tumors can sustain the survival of circulating GMEC, and their subsequent incorporation into the tumor vasculature.

In studies to determine the dose and schedule of *lacZ*-GMEC administration, we observed that over 90% of lung metastases were targeted after three i.v. injections of 10^5 GMEC, but not after a single dose of a larger inoculum (Table 3 vs. Table 4, Figure 2C vs. Figure 3). One explanation for the poor tumor targeting following a single GMEC administration is the heterogenous angiogenic behavior of the individual tumor metastasis. That is, in certain instances the i.v.-injected *lacZ*-GMEC seeded growing tumors at an optimal time for incorporation and growth within the developing tumor vasculature. Other metastases, perhaps too small to sustain active angiogenesis,[24] may not promote GMEC incorporation and/or survival. In addition, following administration of a single small inoculum, GMEC are unlikely to target every tumor deposit present at the moment of injection. Another possibility is that bolus injection of large numbers of GMEC results in their accumulation in the lung microvasculature. This has been observed in our previous study of systemic GMEC administration, where most of the *lacZ*-expressing endothelial cells were retained by the lungs shortly after tail vein injection.[11] In the current study, we noted GMEC clumping and obstruction of small vessels following intravenous administration at high concentration (10^7 GMEC in 0.1 mL PBS). This probably accounts for the death of the majority of the animals treated with 10^7 *lacZ*-GMEC, as well as the low targeting efficiency of this dose in the survivors. Clearly, optimization of the schedule and concentration of GMEC administration (e.g., slow continuous infusion), as well as the route of administration (e.g., intra-arterial) will have to be explored in future studies.

Another factor affecting the efficiency of GMEC-targeting of tumor metastases is the repetitive seeding of the bloodstream with tumor cells from the primary tumor or tumor cells dislodged from other sites. For these reasons, the kinetics of GMEC administration will determine, to a large extent, both targeting efficiency and extent of GMEC incorporation at a particular tumor deposit. This hypothesis is supported by the finding that the number of detectable pulmonary metastases decreased whereas the *lacZ*-GMEC–targeting efficiency increased by 10% following complete removal of the primary tumor (data not shown). The combination of complete removal of the primary tumor with multiple administration of *lacZ*-GMEC resulted in more than two-fold increase in tumor targeting.

The finding that i.v.-injected GMEC can preferentially accumulate at multiple tumor sites in the lungs and subcutaneous tissue led us to investigate the therapeutic utility of this novel approach. The results of our studies in the experimental metastasis model demonstrated that systemic administration of GMEC constitutively expressing a rhIL-2 transgene can mediate the regression of established tumor metastases and prolonged the survival of the tumor-bearing animals. Administration of GMEC with a control vector (*neo*-GMEC) did not elicit any reduction in tumor burden, suggesting that the tumor regression was mediated by rhIL-2 expression, presumably by stimulation of a local antitumor effector cells.[24,32] Of interest, the administration of rhIL-2–secreting GMEC did not produce any observable toxicity, such as body weight loss or lethargy, as has frequently been reported in animals after systemic administration of IL-2 therapy.[32] In contrast, others have reported that i.v.-administered NIH3T3[16] or B16F10 melanoma cells[45] transduced to express IL-2 were unable to significantly reduce the rate of tumor growth. Because only a fraction of i.v.-administered cells would be expected to lodge at the site of tumors, the difference in these results may be related, in part, to the fact that the IL-2-GMEC, but not the IL-2/NIH3T3 or lethally irradiated IL-2/B16F10 used in the

other studies, proliferated in response to the angiogenic factors at the sites[11] (Fig 2C), and, therefore, were able to attain the requisite number cells to produce levels of IL-2 that were sufficient to elicit an antitumor effect. Also, the capacity of the GMEC to readily participate in tumor-associated blood vessels formation (Fig 2, A and C), whereas NIH3T3 and B16F10 melanoma cells do not, may have permitted longer survival of the GMEC, and thus, expression of more IL-2 at the local tumor sites. Moreover, the strategic location of the GMEC at the interface of blood and tumor cells possibly accounted for enhanced activity of the expressed recombinant IL-2, thereby inducing a more effective antitumor immune response.

Nonendothelial cells such as lymphocytes, dendritic cells, and fibroblasts can be genetically modified, and are capable of expressing exogenous gene(s) at tumor sites.[55–59] However, infusion of lymphocytes in patients has been associated with serious side effects,[32,57] and fibroblasts have been reported to be incapable of maintaining long-term survival or expression of exogenous genes *in vivo* in the absence of a matrix scaffold.[55] The results of the present study demonstrate for the first time that targeted delivery of therapeutic gene–expressing endothelial cells to sites of tumor-associated angiogenesis is a feasible, efficient, safe, and potentially effective strategy to treat established tumor metastases in the lungs.

Our finding that fewer X-gal–positive cell clusters were seen at the subcutaneous tumor sites compared to the metastatic foci in the lungs suggests that the lung may be filtering out some of the circulating GMEC. Intra-arterial administration of GMEC using a catheter-based delivery system to by-pass the lung should eliminate this problem. The use of a catheter-based system would also permit selective infusion of specific organs with GMEC in the clinical setting.

Several reports have shown that hematopoietic stem/progenitor cells from human, dogs, mice, and rabbits can differentiate into cells expressing endothelial cell–specific makers *in vitro* and *in vivo*.[12,60–65] In a number of these studies, Asahara et al,[12,61] Takahashi et al,[62] Schatteman et al,[63] and Kalka et al[64] also demonstrated that endothelial cells derived from these precursor cells incorporate into neovasculature induced by wound healing, ischemia, corpus luteum, or tumorigenesis, while continuing to stably express foreign genes. Furthermore, when genetically modified to express a soluble, truncated form of the vascular endothelial growth factor receptor Flk-1/KDR, endothelial precursor cells produced the recombinant Flk-1 protein that inhibited the migration of human umbilical vein endothelial cells *in vitro* and the growth of murine neuroblastoma cells in syngeneic mice *in vivo*.[65] These findings indicate that hematopoietic stem/progenitor cells may serve as sources of endothelial cells and that endothelial cells derived from these sources can be genetically engineered to deliver therapeutic molecules that would inhibit tumor growth as we have observed in our study. Therefore, in clinical application of our strategy, endothelial cells might be isolated from the patient's subcutaneous fat, bone marrow, or derived from stem/progenitor cells. We are currently investigating the *in vivo* distribution of endothelial cells from these sources, as well as their ability to target and abrogate tumors in organs where metastases frequently occur, including bone, brain, lungs, and liver.

In summary, our studies show that angiogenic factors elaborated by tumor metastases can mediate their specific targeting by intravenously administered GMEC *in vivo*. These results suggest that the administration of endothelial cells genetically modified to express therapeutic transgenes is a potentially safe and effective strategy for the treatment of metastatic disease.

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